Yeast histone deposition protein Asf1p requires Hir proteins and PCNA for heterochromatic silencing

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Background: Position-dependent gene silencing in yeast involves many factors, including the four *HIR* genes and nucleosome assembly proteins Asf1p and chromatin assembly factor I (CAF-I, encoded by the *CAC1–3* genes). Both *cac*Δ *asf1*Δ and *cac*Δ *hir*Δ double mutants display synergistic reductions in heterochromatic gene silencing. However, the relationship between the contributions of *HIR* genes and *ASF1* to silencing has not previously been explored.

Results: Our biochemical and genetic studies of yeast Asf1p revealed links to Hir protein function. In vitro, an active histone deposition complex was formed from recombinant yeast Asf1p and histones H3 and H4 that lack a newly synthesized acetylation pattern. This Asf1p/H3/H4 complex generated micrococcal nuclease–resistant DNA in the absence of DNA replication and stimulated nucleosome assembly activity by recombinant yeast CAF-I during DNA synthesis. Also, Asf1p bound to the Hir1p and Hir2p proteins in vitro and in cell extracts. In vivo, the *HIR1* and *ASF1* genes contributed to silencing the heterochromatic *HML* locus via the same genetic pathway. Deletion of either *HIR1* or *ASF1* eliminated telomeric gene silencing in combination with *pol30–8*, encoding an altered form of the DNA polymerase processivity factor PCNA that prevents CAF-I from contributing to silencing. Conversely, other *pol30* alleles prevented Asf1/Hir proteins from contributing to silencing.

Conclusions: Yeast CAF-I and Asf1p cooperate to form nucleosomes in vitro. In vivo, Asf1p and Hir proteins physically interact and together promote heterochromatic gene silencing in a manner requiring PCNA. This Asf1/Hir silencing pathway functionally overlaps with CAF-I activity.

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Received: **20 December 2000**
Revised: **7 February 2001**
Accepted: **12 February 2001**

Published: **3 April 2001**

Current Biology 2001, 11:463–473

0960-9822/01/$ – see front matter

Background

Synthesis of the four core histones (H2A, H2B, H3, and H4) and their assembly into nucleosomes is coordinated with chromosome replication in eukaryotes [1–3]. Several histone chaperone proteins that deliver newly synthesized histones to DNA have been described. One of these proteins, chromatin assembly factor I (CAF-I), is a heterotrimeric protein complex whose biochemical activity and primary structure are conserved from budding yeast to humans [4, 5]. CAF-I performs the first step of nucleosome formation, deposition of the histone $(H3/H4)_2$ tetramer onto DNA [6, 7]. CAF-I delivers histones to DNA molecules that have been recently replicated either bidirectionally or during DNA repair [8, 9]; it is targeted to replicated DNA via a direct interaction with proliferating cell nuclear antigen (PCNA), the DNA polymerase processivity factor [10].

Budding yeast cells lacking any of the three genes (*CAC1*, *CAC2*, or *CAC3*) encoding CAF-I subunits display increased sensitivity to ultraviolet irradiation [5, 11] and reduced position-dependent gene silencing at telomeres [5, 12, 13], the silent mating loci [14, 15], and ribosomal DNA [16]. Gene silencing at these loci in yeast is a chromatin-mediated process analogous to heterochromatic silencing in multicellular organisms; mutations in histone termini [17, 18] or histone binding heterochromatin proteins [19] disrupt this silencing.

Histone gene transcription is repressed outside of the G1/S transition in yeast. This cell cycle–dependent repression requires the histone regulatory (*HIR*) genes, *HIR1*, *HIR2*, *HIR3*, and *HPC2* [20, 21], through a mechanism likely involving the modulation of specialized chromatin structures [22]. Mutations in *HIR* genes have minor effects on silencing at telomeres and the silent mating loci [14], and the growth of *S. cerevisiae* cells is not affected when either *CAC* genes or *HIR* genes are mutated. However, *cac*Δ *hir*Δ double-mutant strains display synergistic reduction of position-dependent gene silencing at both telomeres and the silent mating loci and exhibit slow growth after germination, increased sensitivity to methyl methanesulfonate, and increased Ty element transposition [14, 23]. These phenotypes occur regardless of which of the three *cac*Δ

or four *hir*Δ gene deletions are combined, and this finding suggests that the Cac and Hir proteins are active as complexes ([14] and J. A. S. and P. D. K., unpublished data). Thus, Hir proteins and CAF-I are important for normal growth and silencing, and their roles partially overlap.

The budding yeast *ASF1* gene was identified in two independent genetic screens for high-dosage disrupters of position-dependent gene silencing [24, 25]. The *Drosophila* homolog of yeast Asf1p was purified in a complex with acetylated forms of histones H3 and H4 and stimulates the nucleosome assembly activity of CAF-I in vitro [26]. Also, CIA protein, a human ASF1 homolog, and yeast Asf1p have recently been shown to bind histones and deposit them on DNA independently of replication [27, 28]. Yeast *asf1*Δ mutants display minor defects in heterochromatic gene silencing [24, 25]. However, *cac*Δ *asf1*Δ double mutants display synergistic reductions in heterochromatic gene silencing, and these results are consistent with the in vitro synergy data [26].

We demonstrated here that the histone binding and deposition activity of Asf1p is independent of a newly synthesized pattern of acetylation, and we detected interactions between Asf1p and the Hir1p and Hir2p proteins in vitro and in cell extracts. *ASF1* and *HIR1* genes contributed to silencing at the *HML* locus via the same genetic pathway. The deletion of either *ASF1* or *HIR1* led to similar telomeric silencing phenotypes upon combination with silencing-defective alleles of the *POL30* gene encoding PCNA. Together, these data suggest that Asf1p and Hir proteins function together for heterochromatin silencing in a manner directed by PCNA.

Results

Assembly of an Asf1p/histone H3/histone H4 complex

To test whether the biochemical synergy between *Drosophila* CAF-I and ASF1 has been evolutionarily conserved and to allow study of the corresponding yeast proteins at a biochemical level, we developed recombinant sources of yeast CAF-I and Asf1p. The *Drosophila* RCAF protein complex that was isolated from embryo extracts consists of ASF1 protein bound to acetylated histone H3/H4 molecules [26]. The RCAF-associated histones are specifically acetylated on H3 lysine 9 and H4 lysines 5 and 12. This pattern is observed on newly synthesized molecules [29]. However, it is unknown whether this modification pattern is important for the in vitro stimulation of *Drosophila* CAF-I. By assembly of an Asf1p/histone complex in vitro, we sought to test whether Asf1p required this histone acetylation pattern in order to function as a histone deposition protein.

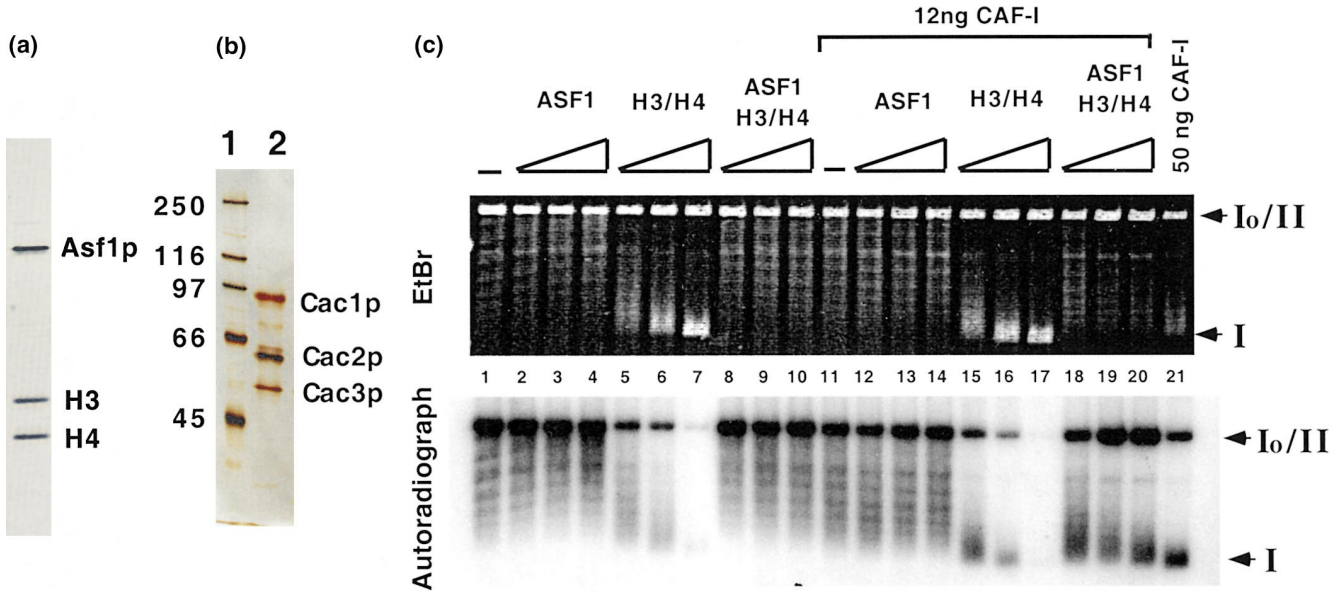
Yeast Asf1p containing six N-terminal histidine residues was overproduced in bacteria and then purified by metal-affinity chromatography. This Asf1p preparation was then further purified by gel filtration chromatography, in some cases after binding to purified human histones H3/H4 (Figure 1a; also see the Supplementary material available with the electronic form of this article on the internet). The histones used in these experiments were purified from human tissue culture cell chromatin and therefore did not carry the newly synthesized acetylation pattern in which histone H4 is diacetylated on lysines 5 and 12 ([29]; see Supplementary material). These data indicate that a specific newly synthesized pattern of acetylation is not required for Asf1p binding.

Asf1p/H3/H4 stimulated nucleosome assembly by CAF-I

Recombinant yeast CAF-I was produced in baculovirus-infected insect cells (Figure 1b) and was shown to stimulate nucleosome assembly in an in vitro SV40-based DNA replication assay (Figure 1c; also see Supplementary material). In this assay, a DNA template containing the SV40 origin of DNA replication was incubated with human cell cytosolic extract containing DNA replication enzymes and supplemented with the viral replication initiation protein T antigen. To determine whether the observed increased superhelicity of the DNA replication products was due to nucleosome formation, we used micrococcal nuclease (MNase) for the digestion of the replication products. MNase preferentially cleaves linker DNA between nucleosomes. The resulting ladder of MNase-resistant bands spaced approximately 160 bp apart on templates replicated in the presence of recombinant yeast CAF-I (Figure 2a, lanes 6–15) confirmed that recombinant yeast CAF-I stimulated nucleosome assembly.

Drosophila ASF1/H3/H4 complexes stimulate substoichiometric amounts of *Drosophila* CAF-I to supercoil replicated templates efficiently [26]. We therefore tested whether yeast Asf1p performed the same reaction and whether this superhelicity reflected nucleosome formation. The addition of Asf1p to the replication assay in the absence of bound histones did not yield supercoiled products (Figure 1c, lanes 2–4), nor did addition of the purified Asf1p/H3/H4 complex (Figure 1c, lanes 8–10). The addition of an equivalent amount of histones H3/H4 alone resulted in a dramatic change in the migration of the template (Figure 2b). This change was concomitant with the inhibition of DNA synthesis (Figure 1c, lanes 5–7), and these results represented nonspecific electrostatic interactions between the histones and DNA. In contrast, the combination of a substoichiometric amount of CAF-I and the Asf1p/H3/H4 complex resulted in greater template supercoiling than that observed with either component alone (Figure 1c, lanes 18–20). Thus, the binding of histones to Asf1p prevented nonspecific histone-DNA interactions and instead promoted synergistic histone deposition by Asf1p and CAF-I.

Figure 1

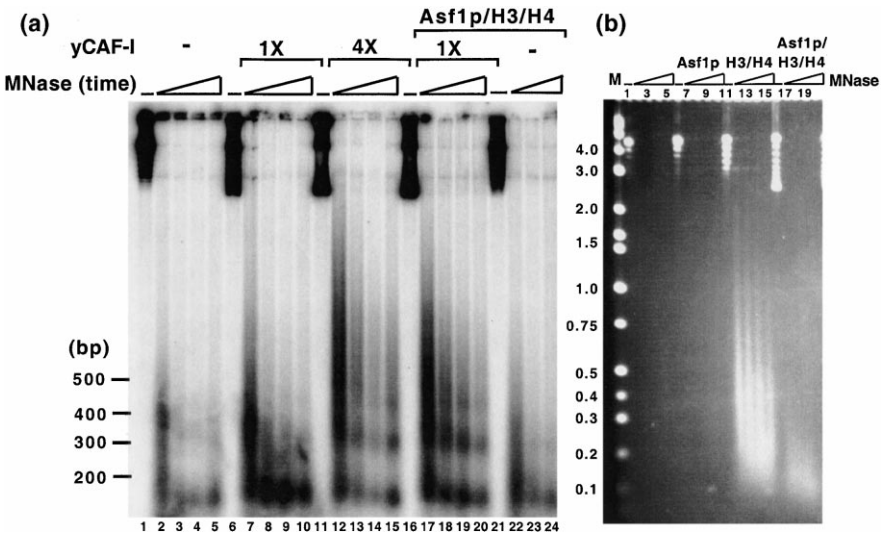


Synergistic activity of recombinant yeast CAF-I and Asf1p/H3/H4 complexes. **(a)** Purified Asf1p/H3/H4 complex. His_6-Asf1p (85 μg, 2.7 nmol) partially purified by metal affinity chromatography was incubated with 60 μg of histones H3 and H4 (2.3 nmol each) and applied to a 43 ml sephacryl S-200HR column. The Asf1p/H3/H4 complex shown here eluted with an apparent molecular weight of approximately 130 kDa and was visualized by Coomassie blue staining of an 18% SDS-polyacrylamide gel. See the Supplementary material for more details. **(b)** Purified recombinant yeast CAF-I. Yeast CAF-I (0.24 μg) purified from baculovirus-infected insect cells was analyzed on a 12.5% SDS-polyacrylamide gel and visualized by silver staining (lane 2). Molecular weight markers (kDa) are indicated (lane 1). **(c)** Stimulation of yeast CAF-1–dependent template supercoiling by Asf1p/H3/H4. Asf1p alone (0.6, 1.2, and 2.4 pmol, respectively, in lanes 2–4 and 12–14), histones H3/H4 alone (0.6, 1.2, and 2.4 pmol, respectively, in lanes 5–7 and 15–17), or the Asf1p/H3/H4 complex (0.6, 1.2, and 2.4 pmol, respectively, in lanes 8–10 and 18–20) were added to 25 μl SV40 in vitro replication assays containing 26 fmol pSV011 DNA molecules (50 ng). Reactions were performed in the absence (lanes 1–10) or presence of low levels of yeast CAF-I (12 ng [71 fmol]/25 μl reaction). As a positive control for assembly, a high level of yeast CAF-I (50 ng [0.30 pmol]/25 μl reaction) alone was added to the SV40 in vitro replication reaction (lane 21).

Previous characterization of the biochemical synergy between *Drosophila* CAF-I and ASF1 proteins demonstrated that those proteins increased the supercoiling of template DNA, but it did not directly demonstrate increased nucleosome formation [26]. To determine definitively whether the increased DNA supercoiling generated by yeast CAF-I and Asf1p/H3/H4 resulted from increased nucleosome formation, we used MNase for the digestion of these replication products. In the presence of substoichiometric amounts of CAF-I (Figure 2a, lanes 6–10), an increased number of mononucleosomes was formed as compared to the background of MNase protection observed either in the absence of any added assembly factor (Figure 2a, lanes 1–5) or with Asf1p/H3/H4 alone (Figure 2a, lanes 21–24). In contrast, in the presence of both assembly factors (Figure 2a, lanes 16–20), more spaced, nuclease-protected species were observed, as indicated by the more-intense disome and trisome bands. Addition of 4-fold more CAF-I in the absence of the Asf1p/H3/H4 complex also resulted in the formation of disomes and trisomes (Figure 2a, lanes 11–15). This observation is consistent with the supercoiling assay (Figure 1c). Together, these data confirm that the increased template superhelicity observed upon combination of CAF-I and Asf1p/H3/H4 resulted from nucleosome assembly.

To test whether Asf1p was able to deposit histones in the absence of DNA synthesis and cell extract, we incubated various purified proteins with a nonreplicating DNA template and analyzed the products by MNase digestion (Figure 2b). As expected, the nuclease rapidly degraded the naked DNA template, and a similar pattern was observed in the presence of Asf1p alone (Figure 2b, lanes 1–10). In the absence of Asf1p, histones H3 and H4 generated a heterogeneous smear of protected DNA species, and this finding is consistent with the histones' ability to inhibit DNA replication and to form rapidly migrating DNA species (Figure 1c, lanes 5–7 and 15–17). In contrast, the Asf1p/H3/H4 complexes generated nuclease-protected species approximately 0.1 kb in length. This result is consistent with the deposition of the histones onto DNA. Thus, Asf1p attenuated nonspecific histone-DNA interactions. Because the Asf1p/H3/H4 complexes used in these experiments were not formed with newly synthesized

Figure 2



Histone deposition by CAF-I and Asf1p/H3/H4 complexes. **(a)** MNase digestion of DNA replication products. SV40 replication reactions (140 μl, containing 146 fmol [280 ng] DNA template) were supplemented with either no assembly factors (lanes 1–5), 67 ng (0.4 pmol) of yeast CAF-I (lanes 6–10 and 16–20), or 0.27 μg (1.6 pmol) of yeast CAF-I (lanes 11–15). In addition, 560 ng of a purified complex of Asf1p and histones H3 and H4 was added to the reactions in lanes 16–24. Reaction products were divided into 25 μl aliquots and digested with 0.2 units of MNase I (Sigma) for 0, 10, 20, 30, and 40 min, and recovered DNA was analyzed on a 2% agarose gel. An autoradiograph of the gel is shown. Molecular mass marker positions and size in base pairs (bp) are indicated on the left. **(b)** MNase protection of DNA by Asf1p/H3/H4 in the absence of DNA replication. Relaxed plasmid DNA molecules (0.30 pmol, 585 ng) were added to no additional protein (lanes 1–5) or to 32 pmol of either Asf1p (lanes 6–10), histones H3/H4 (lanes 11–15), or Asf1p/H3/H4 complexes (lanes 17–20). Reaction products were divided into five aliquots and digested with 0.2 units of MNase I for 0 min (lanes 1, 6, 11, 16), 5 min (lanes 2, 7, 12, 17), 10 min (lanes 3, 8, 13, 18), 20 min (lanes 4, 9, 14, 19), or 40 min (lanes 5, 10, 15, 20). Recovered DNA was separated on a 2% agarose gel and visualized by ethidium bromide staining. (M) DNA size markers in base pairs are indicated.

histones (see Supplementary material), Asf1p is able to deposit histones lacking that specific pattern of acetylation. Also, we note that histone deposition by the Asf1p/H3/H4 complex occurred in the absence of other proteins (Figure 2b) but was not efficient in replication reactions in the absence of CAF-I (Figure 1c). We therefore hypothesize that other factors in the human cell extract may modulate the activity of Asf1p.

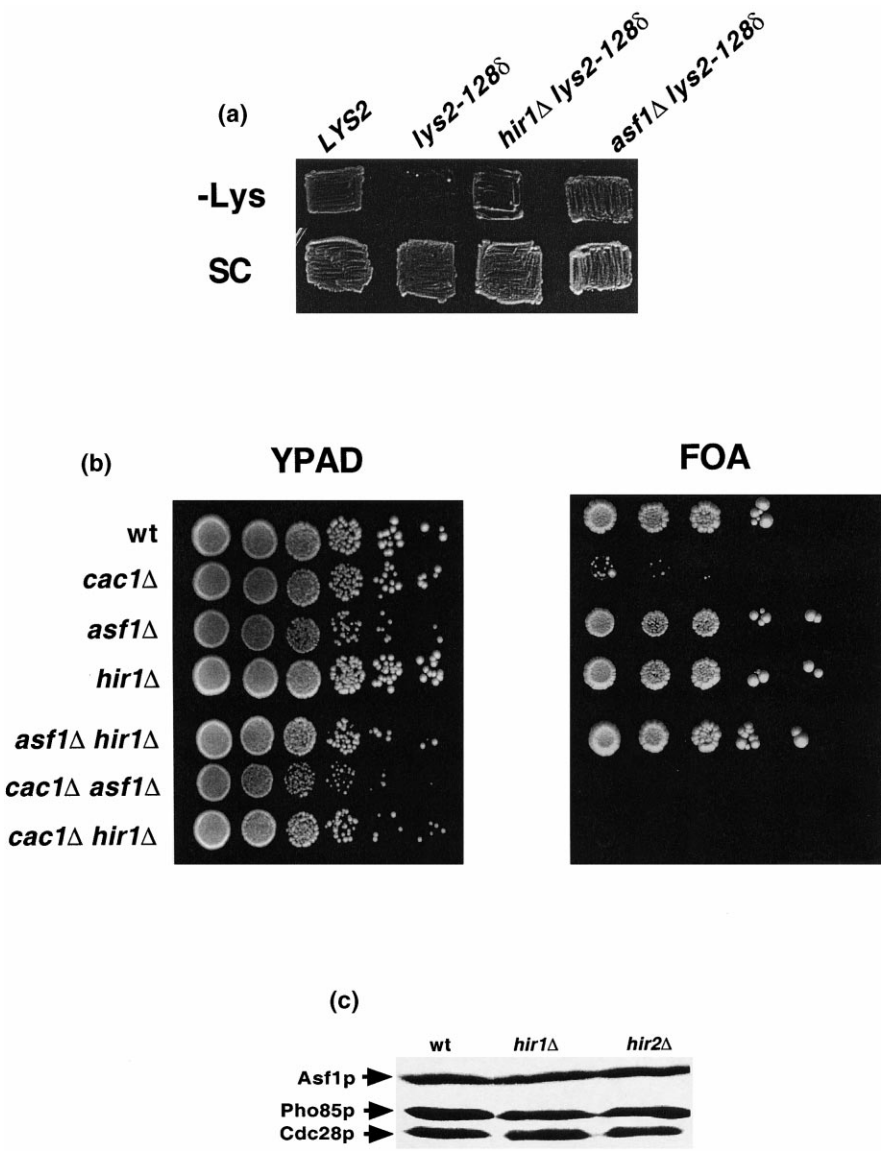
Gene expression and silencing phenotypes caused by *asf1*Δ and *hir*Δ deletions

Asf1p cooperates with CAF-I to silence genes adjacent to yeast telomeres [26]. Because this genetic synergy is similar to that observed between *CAC* and *HIR* genes [14, 23], we tested whether other phenotypes displayed by *hir*Δ strains were observed in *asf1*Δ cells. We first examined the Spt (Supressor of Ty) phenotype because all *hir*Δ strains display a Spt$^-$ phenotype [21, 30]. The Spt phenotype is assessed by the examination of the expression of genes in which the promoter is separated from its coding sequences by Ty transposable elements [31]. In Spt$^+$ (wild-type) cells, the intervening Ty sequences block transcriptional elongation into the coding region and thus eliminate or reduce gene function. In contrast, the mutation of *SPT* genes results in the activation of a cryptic transcriptional start site within transposon DNA and thus restores gene function. Notably, many of the genes identified in screens for *spt* mutants encode proteins that regulate transcriptional activity in the context of chromatin [32, 33]. For example, *SPT3*, *SPT8*, and *SPT20* encode components of the histone acetyltransferase–containing SAGA complex (reviewed in [33]), *SPT5* and *SPT6* are involved in transcriptional elongation [34], and *SPT10* and *SPT11* are histone genes themselves [35]. Furthermore, the *spt1* mutation proved to be an allele of the *HIR2* gene [30].

We assessed the Spt phenotype by using the *lys2–128*δ allele, which causes lysine auxotrophy in otherwise wild-type strains because of a transposon insertion in the *LYS2* gene promoter [36]. Strains containing both the *lys2–128*δ gene and a *hir1*Δ deletion displayed an Spt$^-$ phenotype as indicated by growth in the absence of lysine, demonstrating that *LYS2* expression has been restored (Figure 3a; [30]). Notably, *asf1*Δ mutants displayed the Spt$^-$ phenotype, as has been shown previously for *hir1*Δ, *hir2*Δ, *hir3*Δ, and *hpc2*Δ mutants [21, 30]. In contrast, the *cac1*Δ deletion did not cause the Spt$^-$ phenotype ([14] and data not shown).

We sought to determine whether *ASF1* and *HIR1* contribute to position-dependent silencing in *cac1*Δ cells by distinct genetic mechanisms. However, at telomeres, both *cac*Δ *hir*Δ and *cac*Δ *asf1*Δ cells display such severe defects in silencing that epistasis analysis was precluded [14, 26]. Therefore, we measured silencing at the more strongly repressed *HML* locus. As reported previously, the silencing of *HML*, as measured by the mating efficiency of *MAT***a** cells, was not affected in *cac1*Δ, *hir1*Δ, or *asf1*Δ cells [14, 24, 25]. In contrast, silencing was reduced approximately 10-fold in *cac1*Δ *hir1*Δ cells (Table 1; [14]). *cac1*Δ

Figure 3

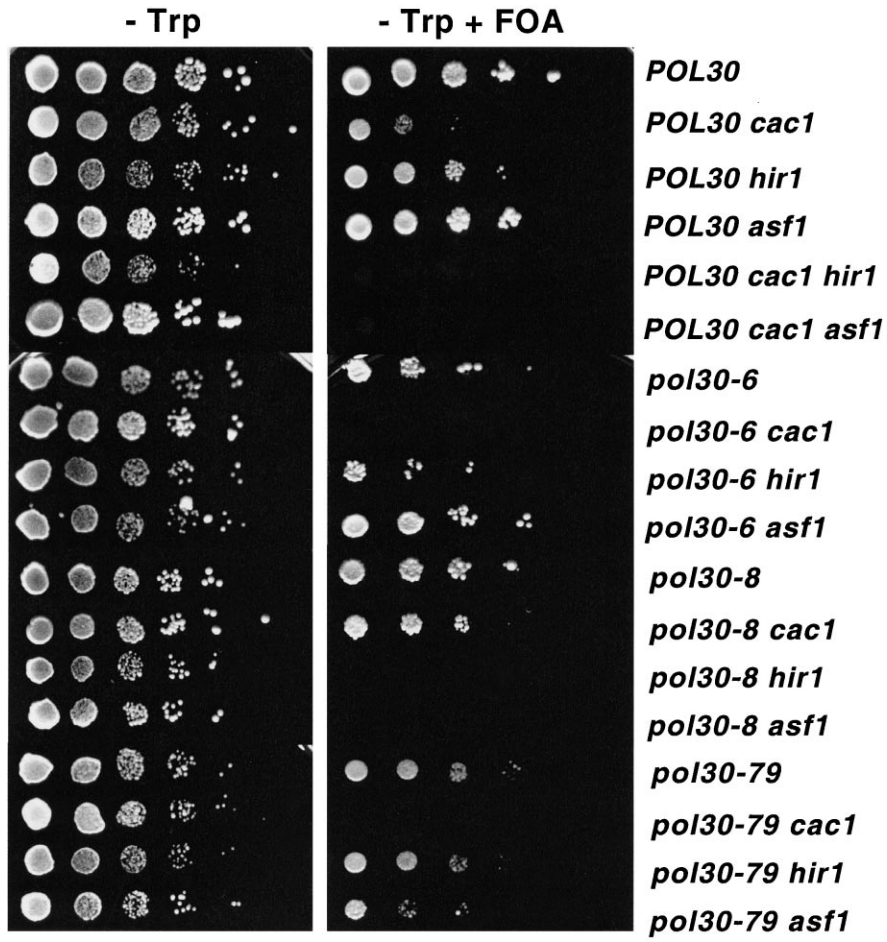


Similar silencing and gene expression phenotypes caused by *hir1*Δ and *asf1*Δ mutations. **(a)** Spt⁻ phenotype of *asf1*Δ mutant cells. Patches of strains PKY028 (wild type), PKY917 (*lys2–128*δ), PKY913 (*hir1*Δ *lys2–128*δ), and PKY951 (*asf1*Δ *lys2–128*δ) were grown on synthetic complete (SC) media or on synthetic media lacking lysine (−Lys) at 30°C for 4 days and photographed. The growth of cells containing the *lys2–128*δ allele in the absence of lysine indicates the Spt⁻ phenotype. **(b)** Telomeric silencing phenotypes. From top to bottom, log-phase cells of yeast strains with PKY numbers 090, 638, 117, 993, 632, 969, and 995 were serially diluted and plated onto rich media (YPAD) as a control for cell number or onto media for the assessment of the extent of silencing of the telomeric *URA3-VIIL* marker gene (FOA). Relevant genotypes are indicated. **(c)** *HIR* gene deletions do not affect Asf1p levels. We separated total cell extracts from strains PKY1041, PKY1140, and PKY1142 on a 12.5% SDS-PAGE gel, transferred them to nitrocellulose, and probed them with anti-FLAG antibodies (Sigma) to detect Asf1p-FLAG protein. As a loading control, the same blot was reprobed with anti-PSTAIRE antibodies (Santa Cruz), which recognize Cdc28p and Pho85p.

*asf1*Δ cells also displayed reduced mating of similar magnitude to the reduction in the *cac*Δ *hir*Δ cells (Table 1). These data were consistent with published work [26] in that the silencing defect of *cac1*Δ *asf1*Δ cells was greater than that observed for the single mutants. However, contrary to previous experiments [26], we observed that *cac1*Δ *asf1*Δ cells retained significant mating ability. Notably, *cac1*Δ *hir1*Δ *asf1*Δ triple-mutant cells mated at frequencies statistically indistinguishable from *cac*Δ *hir*Δ and *cac*Δ *asf1*Δ mutant cells. Furthermore, mating frequencies of *hir1*Δ *asf1*Δ cells were statistically indistinguishable from those observed in wild-type and *hir1*Δ and *asf1*Δ single-mutant cells (Table 1), and *hir1*Δ *asf1*Δ cells displayed wild-type levels of telomeric silencing (Figure 3b). Together, these data demonstrate that *HIR* genes and *ASF1* contribute to position-dependent silencing by the same genetic pathway. Furthermore, Asf1 and Hir proteins together functionally overlap with CAF-I to generate silenced heterochromatin.

CAF-I is recruited to replicated DNA via a direct interaction with PCNA, and mutation of PCNA in both yeast and *Drosophila* leads to defects in heterochromatic gene silencing [10, 37, 38]. We predicted that PCNA mutations defective in recruitment of CAF-I to heterochromatic loci would require Asf1p and Hir proteins for silencing; conversely, any PCNA mutations that prevent Asf1p/Hir proteins from contributing would depend on CAF-I function for silencing. To test these predictions, we determined the effects of PCNA mutations on telomeric silencing in *hir1*Δ and *asf1*Δ strains. For these experiments, the chromosomal *POL30* gene encoding PCNA was deleted,

Figure 4



Mutations in PCNA prevent the contribution of Asf1p/Hir proteins to telomeric silencing. From top to bottom, log-phase cells of yeast strains with PKY numbers 800, 802, 2093, 1644, 1690, 1646, 1890, 1937, 1939, 1888, 1893,1940,1942,1895, 836, 837, 1730, and 1734 were serially diluted and plated onto synthetic media lacking tryptophan (−Trp) as a control for cell number or onto the same media with FOA (−Trp + FOA) for the assessment of the extent of silencing of the telomeric *URA3-VIIL* marker gene. Relevant genotypes are indicated.

and cells were maintained with either wild-type or mutant versions of the *POL30* gene on a low-copy plasmid. In the presence of a wild-type *POL30* gene, *cac1*Δ cells displayed reduced telomeric silencing, *hir1*Δ and *asf1*Δ cells displayed no observed silencing defects, and *cac1*Δ *hir1*Δ and *cac1*Δ *asf1*Δ double mutants displayed extremely low levels of silencing. These results are consistent with published data (Figure 4; [5, 14, 26]. We also examined three *pol30* mutations known to affect silencing. First, a double point–mutant allele known as *pol30–8* causes defects in silencing that are similar in magnitude to the effects of deleting the *CAC1* gene; silencing at telomeres and *HMR* is reduced in *pol30–8* cells, and these phenotypes are not exacerbated by the further deletion of *CAC1* [37]. In contrast, we observed that the deletion of either *HIR1* or *ASF1* in combination with the *pol30–8* allele led to dramatic reduction of telomeric silencing. This reduction resulted in a level of silencing comparable to that observed in *cac1*Δ *hir1*Δ *POL30* and *cac1*Δ *asf1*Δ *POL30* cells (Figure 4). We also tested the *pol30-79* allele, which contains two point mutations in the interdomain loop, a region of contact with many proteins [39, 40]. *pol30-79* causes telomeric silencing defects in an otherwise wild-type strain. These defects are exacerbated by the deletion of *CAC1*, and this finding suggests that the Pol30-79p protein contributes to silencing in a manner partially independent of CAF-1 [37]. In contrast to *pol30–8* cells, silencing in *pol30-79* cells was not altered by either *hir1*Δ or *asf1*Δ deletions (Figure 4). The third mutation that we tested was the *pol30-6* allele. As in *pol30-79* cells, silencing in *pol30-6* cells was reduced significantly by the deletion of *CAC1* ([37]; Figure 4b) but not by either *hir1*Δ or *asf1*Δ deletions (Figure 4a). Thus, the deletion of either *HIR1* or *ASF1* resulted in similar effects on both telomeric and *HML* gene silencing. Furthermore, the contribution of Asf1 and Hir proteins to silencing was affected by mutations in PCNA.

The links between *ASF1* and *HIR1* gene function would have been observed if Asf1p synthesis depended on *HIR* gene function (or vice versa). However, immunoblot analysis demonstrated that the steady-state levels of the Asf1p protein remained unchanged in *hir1*Δ and *hir2*Δ mutant cells (Figure 3c). Likewise, the deletion of the *ASF1* gene

did not affect the steady-state levels of *HIR1*, *HIR2*, *HIR3*, or *HPC2* mRNA (data not shown). Together, these data favored models in which Asf1p and Hir proteins functionally interact to contribute to silencing. We therefore tested whether Asf1p and Hir proteins physically interact. Indeed, a glutathione-S-transferase (GST-Asf1p) protein fusion was able to coprecipitate in vitro–translated Hir1p and Hir2p proteins (Figure 5a). We included ethidium bromide in these precipitation experiments to avoid nonspecific interactions mediated by nucleic acids [41]. In contrast, no interaction was detected in a similar assay with Hir3p or Hpc2p, the other two known Hir proteins (data not shown), nor did GST-Asf1p interact with β-galactosidase (Figure 5a). To confirm that these interactions that were observed in vitro can occur in cellular extracts, we performed coimmunoprecipitation experiments. We observed that Hir1p and Hir2p coprecipitated with Asf1p in an epitope- and antibody-dependent manner (Figure 5b). Furthermore, these interactions were observed in extracts treated with RNase, DNase, and ethidium bromide, and this observation supports our hypothesis that they were not an indirect result of interaction with nucleic acids. Together, these data support the hypothesis that Asf1p and Hir proteins interact physically in order to contribute together to heterochromatic silencing.

Discussion

CAF-I/Asf1p synergy in vitro

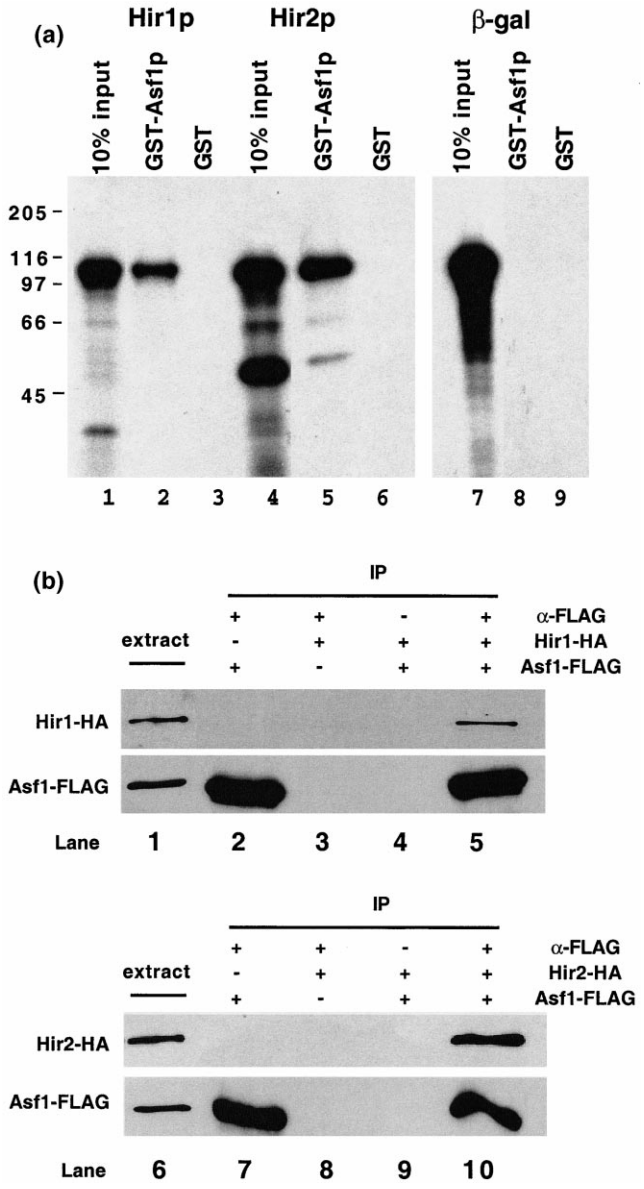
As previously observed for the homologous *Drosophila* proteins [26], the addition of yeast Asf1p/H3/H4 complexes to substoichiometric amounts of yeast CAF-I resulted in increased supercoiling of replicated DNA templates (Figure 1c). By MNase digestion analysis, we demonstrated for the first time that CAF-I and Asf1p supercoiled replicated DNA templates via nucleosome formation (Figure 2a).

In the absence of Asf1p, CAF-I was required in stoichiometric amounts for histone deposition (see Supplementary material). Thus, Asf1p might promote efficient recycling of CAF-I, perhaps by reloading histones onto CAF-I. However, the converse possibility, in which CAF-I reloads histones onto Asf1p, is also consistent with our data. Alternatively, DNA molecules containing a few histone $(H3/H4)_2$ tetramers deposited by substoichiometric levels of CAF-I may be a more efficient substrate for deposition by the Asf1p/H3/H4 complex (or vice versa). Variants of this model entertain scenarios in which Asf1p or CAF-I help recruit other factors to the DNA template.

The role of histone acetylation in nucleosome assembly

An active complex of recombinant Asf1p was formed with human histones purified from somatic cell chromatin (Figures 1 and 2). Thus, the specific pattern of acetylation on newly synthesized histones was not required for the activity of yeast Asf1p in vitro. Similar observations have

Figure 5



Asf1p binds to Hir1p and Hir2p in vitro and in cell extracts. **(a)** ^{35}S-labeled, in vitro–translated Hir1p (lanes 1–3), Hir2p (lanes 3–6), or β-galactosidase (lanes 7–9) was precipitated with approximately 2 μg of glutathione-agarose bound GST-Asf1p (lanes 2, 5, and 8) or unfused GST (lanes 3, 6, and 9). Ten percent of the amount of each labeled protein added to the precipitation reactions was loaded in lanes 1, 4, and 7. **(b)** Hir1p and Hir2p coimmunoprecipitate with Asf1p. Immunoblot analyses of cell extracts and immunoprecipitation reactions are shown. Cell extracts from PKY2178 (lane 1) and PKY2177 (lane 6) represent 1/50th of the amount of protein input for immunoprecipitations. α-FLAG immunoprecipitations (lanes 2–5 and 7–10) were performed on cell extracts from yeast strains expressing the indicated tagged proteins (lanes 2 and 7, PKY1041; lane 3, PKY2061; lanes 4 and 5, PKY2178; lane 8, PKY2062; lanes 9 and 10, PKY2177).

Table 1

Quantitative mating assays.

Strain	Relevant genotype	Mating efficiency
PKY028	wild type	1.0
PKY020	*cac1*Δ	0.93 ± 0.42
PKY087	*hir1*Δ	0.70 ± 0.086
PKY937	*asf1*Δ	0.66 ± 0.27
PKY969	*asf1*Δ*hir1*Δ	1.4 ± 0.44
PKY128	*cac1*Δ*hir1*Δ	0.12 ± 0.059
PKY1019	*cac1*Δ*asf1*Δ	0.16 ± 0.053
PKY971	*cac1*Δ*hir1*Δ*asf1*Δ	0.11 ± 0.047

Mating of *MAT***a** strains was quantified as a measure of the strength of silencing at the *HML* locus. Average mating frequencies ± standard deviations, normalized to the wild-type strain, for five independent mating experiments are presented. The mating frequencies for the wild-type, *cac1*Δ, *hir1*Δ, *asf1*Δ, and *asf1*Δ*hir1*Δ strains are not statistically distinguishable at a 99.9% confidence limit by the Student's t test. Likewise, the mating frequencies of the *cac1*Δ*hir1*Δ, *cac1*Δ*asf1*Δ, and *cac1*Δ *hir1*Δ*asf1*Δ strains are indistinguishable from each other by the same test. In contrast, the mating frequencies of each of these latter three strains are significantly different from those of the first set of strains by the same test.

been made for human CAF-I; in that case, recombinant histones H3/H4 with or without N-terminal tails were deposited by human CAF-I with equal efficiency [42]. Furthermore, nucleosomes lacking tail domains of all four core histones can be assembled in vitro by dialysis [43]. Thus, although synthesis-related N-terminal modifications of histones H3/H4 are found throughout eukaryotic organisms [29], the purpose of this evolutionarily conserved process remains unknown (see [44] for further discussion).

*asf1*Δ and *hir*Δ mutations cause similar chromatin-related phenotypes

As observed for *hir*Δ mutants [21, 30], *asf1*Δ mutants displayed a Spt$^-$ phenotype (Figure 3a). Like *hir*Δ mutants, *asf1*Δ mutants also constitutively express histone gene transcripts during the cell cycle [45]. Thus, in addition to their role in heterochromatin-mediated silencing, Asf1p and Hir proteins affect gene expression at nonsilenced loci.

We demonstrated here that Asf1p and Hir1p contribute to *HML* silencing in the absence of CAF-I via the same genetic pathway (Table 1). Likewise, in the presence of the *pol30–8* allele, which prevents CAF-I from contributing to silencing [37], the deletion of either *HIR1* or *ASF1* greatly reduced telomeric silencing (Figure 4a). Together, these data are consistent with Asf1p and Hir proteins acting in concert to promote nucleosome assembly.

How is histone deposition by the Asf1p-histone complex related to Hir protein function in vivo? Asf1p may serve as an intermediate histone chaperone that delivers its cargo to Hir proteins at heterochromatic loci; the converse is also possible. The first model suggests that Hir proteins are DNA binding proteins, but both models suggest that Hir proteins bind histones. Although it is unclear whether Hir proteins interact directly with DNA, there is genetic evidence in yeast for histone binding by Hir proteins. Hir proteins are required for dosage compensation of histone gene transcription in yeast [46], in which chromosomal copies of the *HTA1-HTB1* locus are repressed upon the overproduction of histones from plasmid-encoded genes. Mutations within the histone N-termini abolish this repression [47], and this finding suggests that Hir proteins are histone binding proteins. However, this dosage compensation involves histones H2A and H2B rather than H3 and H4, so it will be necessary to establish which of the four core histones, if any, are bound by Hir proteins in vivo. Biochemical analysis suggests that the HIRA protein, a mammalian Hir homolog, does bind to histone H2B in cellular extracts [48].

However, in vitro, Asf1p itself can interact with DNA and histones in the absence of Hir proteins. For example, yeast Asf1p directly delivers histones H3/H4 to DNA in the absence of other proteins (Figure 2b; [28]); similar results have been reported for a human homolog of Asf1p fused to GST [27]. Therefore, it appears likely that Asf1p directly interacts with DNA templates during nucleosome assembly. In vivo, Hir proteins might be required for aspects of histone deposition by Asf1p that are not recapitulated in the in vitro assays — for example, for reloading of histones onto Asf1p or for locus-specific targeting. Asf1p bound to two Hir proteins in cell extracts and in vitro (Figure 5), and these results are consistent with there being a direct, functional interaction among these proteins.

Our genetic data supports a model in which CAF-I and Asf1p/Hir proteins comprise functionally overlapping but distinct nucleosome assembly activities that contribute independently to heterochromatic silencing. However, our biochemical data demonstrate that CAF-I and Asf1p function synergistically to form nucleosomes. There are several possible explanations for this discrepancy between the in vivo and in vitro data. For example, the CAF-I/Asf1p synergy observed in vitro may not apply in vivo at heterochromatic loci, but it may be important at other genomic locations. Alternatively, Asf1p may assist nucleosome assembly by both CAF-I and Hir proteins in vivo, but only the Asf1p-Hir interactions may have a consequence for silencing. It is also possible that CAF-I and Asf1p contribute to silencing in vivo in a manner distinct from histone deposition, for example by recruiting other silencing proteins to heterochromatin. Furthermore, it is likely that many forms of biological regulation are not recapitulated in the existing biochemical assays. For instance, the histone binding and deposition activity of Asf1p has recently been shown to be regulated by the DNA damage checkpoint kinase Rad53p [28].

PCNA is involved in Asf1/Hir protein–medicated silencing

The *pol30–8* mutation abolishes the contribution of CAF-I to telomeric silencing [37]. In *pol30–8* cells, the deletion of either *HIR1* or *ASF1* eliminated residual telomeric silencing (Figure 4b). This finding is consistent with the notion that CAF-I and Asf1/Hir proteins represent two different yet functionally overlapping heterochromatin-forming activities. Conversely, the *pol30-6* and *pol30-79* mutations cause synergistic silencing defects with *cac1*Δ deletions [37]. These mutations do not cause synergistic silencing phenotypes with *hir1*Δ and *asf1*Δ deletions (Figure 4b). These data suggest that the *pol30-6* and *pol30-79* mutations affect the ability of Hir and/or Asf1 proteins to contribute to silencing. Thus, PCNA appears to be central to both CAF-I– and Hir/Asf1p-based contributions to silencing.

The *pol30-79* allele contains two point mutations in the interdomain loop of PCNA. This loop is a conserved site of interaction with a variety of proteins, including the p21 cell cycle inhibitor, the flap endonuclease FEN-1, and the XPG protein required for incision during nucleotide excision repair [40, 49]. Interestingly, neither Asf1p nor any of the four Hir proteins contain the canonical PCNA interdomain loop binding motif, Qxx(h)xx(a)(a), where (h) represents hydrophobic residues and (a) represents aromatic residues [40]. Several possibilities could explain the lack of this motif on Asf1p and Hir proteins. For example, the interaction between PCNA and Asf1p and/or Hir proteins could occur via an unrecognized primary-sequence motif; alternatively, the interaction could be indirect and bridged by another protein. Both the *pol30-6* and *pol30-79* alleles caused similar phenotypes in our experiments (Figure 4b); however, these alleles change different PCNA amino acids to alanines (*pol30-6*: amino acids 41 and 42; *pol30-79*: amino acids 126 and 128; [39, 50]). These pairs of residues are not located near to each other in the three-dimensional structure of the protein [50]. Thus, the interaction between the Asf1p/Hir proteins and PCNA is likely to be complex.

Conclusions

An Asf1p/H3/H4 complex functional for histone deposition can be formed with histones lacking a newly synthesized acetylation pattern. Yeast CAF-I and Asf1p cooperate to form nucleosomes in vitro. In vivo, Asf1p and Hir proteins interact and together promote gene silencing at telomeres and the *HML* locus. This Asf1/Hir silencing pathway functionally overlaps with CAF-I activity at these heterochromatic loci. Mutations in the gene encoding PCNA prevent the Asf1/Hir proteins from contributing to telomeric silencing, and this finding suggests that PCNA directs histone deposition by the Asf1/Hir proteins.

Materials and methods

Yeast strains and silencing assays

Strain genotypes and recombinant DNA details are listed in the Supplementary material. Previously described deletion alleles in the W303 background include *cac1*Δ*::LEU2*, *URA3-VIIL* [5], *hir1*Δ*::HIS3, hir2*Δ*::HIS3* [47], and *pol30*Δ*::hisG* [11]. All new deletions were made in strain W303 [52] by single-step gene replacement and were checked for proper insertion by DNA blot hybridization or PCR. The pBL230, pBL230-6, pBL230–8, and pBL230-79 plasmids containing the wild-type and mutant *POL30* alleles were the gift of Peter Burgers [39, 50]. The *lys2–128*δ allele was introduced into the W303 genetic background by seven backcrosses from strain FY120, a gift of Fred Winston [34]. Standard procedures for genetic crosses and tetrad analysis were used [53]. YPAD is YPD medium supplemented with 50 mg/L of adenine. Quantitative mating assays were performed as described [54], with strains 216 (*MAT***a**, *his1*) and 217 (*MAT*α, *his1*) as the testers. For the measurement of telomeric silencing, the *URA3-VIIL*–marked telomere was used ([5]; originally described in [55]). Five μl of 10-fold serial dilutions of log-phase cells adjusted to an initial concentration of A_{600} = 1.0 were spotted onto media with or without FOA. Cells on FOA-free media were photographed after 3 days of growth at 30°C; cells were grown in the presence of FOA for 7 days prior to photography.

Nucleosome assembly assays

The SV40-based DNA replication assay and the micrococcal nuclease digestion of products were performed as described [5]. For nonreplicating-template experiments, plasmid pSV011 [4] was first relaxed with human topoisomerase I in 25 mM Hepes (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT.

GST-fusion protein interaction experiments

Protein overproduction methods are described in the Supplementary material. 10 μl of glutathione-agarose beads bound to GST-Asf1p or GST were mixed with 5 μl of in vitro–translated protein in a total volume of 200 μl 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% NP-40, 25 mM NaCl, and 50 μg/ml ethidium bromide. Proteins were mixed at 4°C for 1 hr, and then beads were collected by centrifugation and washed three times with 1 ml of the same buffer containing 250 mM NaCl. Precipitated proteins were analyzed on a 10% SDS-PAGE gel.

Coimmunoprecipitation of Asf1p and Hir proteins

Yeast cell extracts were prepared essentially as described [56], except that slightly modified buffer conditions allowed for DNase I digestion. Log-phase cultures (25 mL) were pelleted and washed in 5 ml lysis/IP buffer (25 mM Tris-Cl [pH 7.5], 125 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.1% Tween 20, 5% glycerol). Cell pellets were then resuspended in 1 ml lysis/IP buffer containing protease inhibitors (1 mM PMSF, 1 μg/mL E64, 1.1 μg/mL phosphoramidon, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, and 1 μg/mL aprotinin) and lysed with glass beads. Cell extracts were treated with RNase (200 μg/mL) and DNase I (20 μg/mL) at room temperature for 10 min; digestion was terminated by the addition of EDTA (10 mM) and EGTA (5 mM). Ethidium bromide (50 μg/mL) was added prior to the ultracentrifugation of cell extracts (35,000 rpm for 30 min at 4°C). α-FLAG antibodies (Sigma; 5 μg/mL final concentration) were added to 0.5 ml soluble extract and rotated overnight at 4°C. Protein G-sepharose beads (10 μl of 50% slurry) were added. Continued incubation for 2–3 hr at 4°C allowed the collection of immune complexes. Beads were collected by centrifugation, washed four times in lysis/IP buffer, and resuspended in SDS-PAGE loading buffer.

Supplementary material

Two supplementary figures, a supplementary table, and supplementary methodological details are available with this article on the internet at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgements

We thank Diana Starr for GST-Asf1p purification; Fred Winston, Peter Burgers, and Mary Ann Osley for providing strains and plasmids as mentioned; Alexa A. Franco, Ann Kirchmaier, and Jasper Rine for comments; and Ann Sutton and Rolf Sternglanz for comments and communication of results prior to publication. This work was supported by Department of Energy funds awarded to P. D. K. and administered through the Lawrence Berkeley National Laboratory. This work was also supported by National Institutes of Health grant 1 R01 GM55712 and by National Science Foundation grant MCB-9982909.

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